Remarks

Claims 2-14 and 16-33 are pending. Claims 2-7, 9, 17, 22, 25, 28, 30, and 31 have been amended. Claims 1 and 15 have been canceled. Claim 30 has been amended to clarify the claimed device as one comprising biodegradable polyhyroxyalkanoate polymer, wherein the polymer has a molecular weight of between 10,000 and 10,000,000 Daltons and wherein the average molecular mass loss of the polymer decreases 20% to 50% over a ten week time period *in vivo*. Support for the amendment to claim 30 can be found, for example, at page 9, lines 6-8 (molecular weight); and page 55, lines 1-2 (average decrease in molecular mass of between 20% to 50%). Claims 2-7, 9, 17, 22, 25, 28, and 31 were amended to properly depend from an existing independent claim.

Priority

With regard to the Examiner's statements relating to the specification's priority claim, the preliminary amendment, mailed on February 26, 2002, requested that the statement "[T]his application is a divisional of pending prior application U.S. Serial No. 09/535,146 filed March 24, 2000, which claims priority to U.S. Serial No. 60/142,238, filed July 2, 1999, and U.S. Serial No. 60/126,180, filed March 25, 1999" be used as the first paragraph of the specification.

Rejection Under 35 U.S.C. § 112, second paragraph

Claims 12, 15 and 30 were rejected under 35 U.S.C. § 112, second paragraph, as being indefinite. Applicants respectfully traverse this rejection.

The units defined in the Markush group of claim 12 find proper antecedent basis in claim 11 wherein "the ***units*** are incorporated into the polymer backbone with chemical linkages...." (emphasis added). Claim 15 has been canceled to remove any redundancy with claim 13. Claim 30 is amended to be properly independent.

Rejection Under 35 U.S.C. § 102

Claims 1-7, 9-15 and 17-33 were rejected under 35 U.S.C. § 102(b or e) as being anticipated by U.S. Patent No. 5,236,431 to Gogolewski ("Gogolewski"), U.S. Patent No. 6,514,515 to Williams ("Williams1"), or U.S. Patent No. 6,623,749 to Williams *et al.* ("Williams2"). Applicants respectfully traverse this rejection to the extent that it is applied to the claims as amended.

Gogolewski

Gogolewski mentions polyhydroxybutyrate, polyhydroxyvalerate and copolymers of hydroxybutyrate and hydroxyvalerate as preferred materials for fixation devices. However, as the enclosed references illustrate, these polymers do not degrade in less than one year *in vivo* See, for example, Hazari, A *et. al.*, *British J. of Plastic Surgery* (1999), 52, 653-657; Hazari, A. *et. al.*, *Journal of Hand Surgery* (1999), 24B(3), 291-295; and Duvernoy, O. *et al.*, *Thoracic Cardiovascular Surgeon* (1995), 43, 271-274. These references demonstrate that those skilled in the art know that PHB degrades very slowly *in vivo*, typically not for 24-30 months after implantation, and that no known polyhydroxyalkanoate polymer, absent modification, degrades *in vivo* in less than one year. Specifically,

1) Hazari, A. et al., *British Journal of Plastic Surgery* (1999), **52**, 653-657, states in the second paragraph on page 653 that "PHB undergoes hydrolytic degradation and is completely absorbed in 24-30 months."

2) Hazari, A. et al., *Journal of Hand Surgery* (1999), **24B**(3), 291-295, states in the third paragraph on page 291 that "PHB is non-antigenic, biocompatible, easy to handle, has good tensile strength and is completely resorbed within 24 to 30 months by hydrolytic degradation...."

3) Duvernoy, O. et al., *Thoracic Cardiovascular Surgeon* (1995), **43**, 271-274, states on page 273 that the "PHB patch was phagocytosed by macrophages and completely removed within a period of 24-30 months.

Applicants modify their PHAs so they degrade in the desired time period. Gogolewski also does not suggest selecting a polymer having an average molecular mass loss of 20% to 50% over a ten week period. This relatively slow rate of molecular mass loss, *in vivo*, allows for the maintenance of polymer material properties while undergoing biodegradation.

Williams1

Williams1 does not teach one to make or select a *modified* polyhydroxyalkanoate with a degradation rate of less than one year to make a device.

There is nothing in Williams1 to suggest selecting a polymer having an average molecular mass loss of 20% to 50% over a ten week period. This relatively slow rate of

molecular mass loss, *in vivo*, allows for the maintenance of polymer material properties while undergoing biodegradation.

Williams2

Williams2 is directed to the development of a method for the purificaton of PHA, specifically to remove endotoxin from the polyhydroxyalkanoate. It does not teach one to make or select a *modified* PHA with a degradation rate of less than one year.

There is nothing in Williams2 to suggest making or selecting a modified PHA polymer having an average molecular mass loss of 20% to 50% over a ten week period. This relatively slow rate of molecular mass loss, *in vivo*, allows for the maintenance of polymer material properties while undergoing biodegradation.

Rejection Under 35 U.S.C. § 103

Claims 1-7, 9-15 and 17-33 were rejected under 35 U.S.C. § 103(a) as being unpatentable over U.S. Patent No. 5,236,431 to Gogolewski ("Gogolewski"), U.S. Patent No. 6,514,515 to Williams ("Williams1"), or U.S. Patent No. 6,623,749 to Williams *et al.* ("Williams2"). Applicants respectfully traverse this rejection to the extent that it is applied to the claims as amended.

Each of the three references cited by the Examiner under 35 U.S.C. § 103(a), have been discussed in the foregoing section, under "Rejection Under 35 U.S.C. § 102". None of Gogolewski, Williams1, or Williams2 suggest making or selecting a *modified* PHA polymer

having an average molecular mass loss of 20% to 50% over a ten week period. Accordingly, none of the references, alone or in combination, make obvious the claimed subject matter.

Double Patenting Rejection

Claims 1-33 were rejected under the judicially created doctrine of obviousness-type double patenting as being unpatentable over claims 27-39 of U.S. Patent No. 6,514,515 to Williams (assigned to Tepha, Inc.). Applicants respectfully traverse this rejection.

The Applicants submit that this is an improper double patenting rejection. 37 C.F.R. § 1.130 (b) states "[W]hen an application or a patent under reexamination claims an invention which is not patentably distinct from an invention claimed **in a commonly owned patent** with the same or a different inventive entity, a double patenting rejection will be made in the application or a patent under reexamination." (emphasis added). The present application is owned by **Metabolix, Inc.** U.S. Patent No. 6,514,515 is owned by **Tepha, Inc.**

Allowance of claims 2-14 and 16-33 is respectfully solicited.

Respectfully submitted,

Patrea L. Pabst
Reg. No. 31,284

Date: January 20, 2004

HOLLAND & KNIGHT LLP
One Atlantic Center, Suite 2000
1201 West Peachtree Street
Atlanta, Georgia 30309-3400
(404) 817-8473
(404) 817-8588 (Fax)

Certificate of Facsimile Transmission

I hereby certify that this Amendment and Response to Office Action, and any documents referred to as attached therein are being facsimile transmitted on this date, January 20, 2004, to the Commissioner for Patents, U.S. Patent and Trademark Office, P.O. Box 1450, Alexandria, VA 22313-1450.

Peggy Bailey

Date: January 20, 2004

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A NEW RESORBABLE WRAP-AROUND IMPLANT AS AN ALTERNATIVE NERVE REPAIR TECHNIQUE

A. HAZARI, G. JOHANSSON-RUDÉN, K. JUNEMO-BOSTROM, C. LJUNGBERG, G. TERENGHI, C. GREEN and M. WIBERG

From the Blond McIndoe Centre, East Grinstead, UK, the Departments of Anatomy and Hand and Plastic Surgery, Umea University, Umea, and the Department of Research and Development, Astra Tech, Gothenberg, Sweden

Poly-3-hydroxybutyrate (PHB), a bacterial storage product, is available as bioabsorbable sheets and has been used in this study for primary nerve repair. The aim was to assess axonal regeneration following such repair and determine the inflammatory response to PHB.

In 20 adult cats, the transected superficial radial nerve was wrapped in PHB sheets, while primary epineural repair was carried out in the contralateral limb. At 6 and 12 months, the repair sites were assessed immunohistochemically for macrophage infiltration and myelinated axons were counted in the distal nerve.

Mean macrophage counts across the whole width of the nerve in both groups at 6 and 12 months showed no statistically significant difference. Nor was there any significant difference between the two groups at both time-points in axon counts, axon diameter, myelin thickness and g-ratio. There was a statistically significant increase in fibre diameters at 12 months, indicating that fibres were undergoing continuous maturation.

Journal of Hand Surgery (British and European Volume, 1999) 24B: 3: 291–295

INTRODUCTION

Current microsurgical techniques for peripheral nerve repair give functional results that are not always optimal (Terzis, 1990). The need to improve these results has led to the quest for a sutureless method of nerve repair that would cause minimal interference with the internal environment of the injured nerve. The use of adhesives such as cyanoacrylate glue (Ferlic and Goldner, 1965) and fibrin (Moy et al., 1988) have not improved results. Welding tissue with carbon dioxide lasers causes thermal damage to the nerve, which exceeds any benefit gained by the absence of foreign material (Richmond, 1986).

Nerve tubulization offers an alternative method of repair of severed nerves with maximal coaptation and minimal injury. Tubulization has several advantages, as it protects regenerating fibres by reducing invasion and scarring of the nerve, and discourages the formation of neuromas.

Microorganisms are capable of producing a wide range of polymers generated from 3-hydroxypropionic acid, which is widely present in nature. One such homopolymer, poly-3-hydroxbutyrate (PHB), is a natural storage product of bacteria and algae and occurs as discrete granules within the cell cytoplasm. PHB granules are produced from bacterial cultures by fermentation followed by solvent extraction. PHB can also be produced from carbon substrates as diverse as glucose, ethanol, acetane, methane and gaseous mixtures of carbon dioxide and oxygen (Anderson and Dawes, 1990). PHB (Astra Tech, Gothenberg, Sweden) is available in the form of sheets which have been used experimentally in cardiovascular surgery (Duvernoy et al., 1995; Malm et al., 1992; 1994). PHB is non-antigenic, biocompatible, easy to handle, has good tensile strength and is completely resorbed within 24 to 30 months by hydrolytic degradation (Gogolewski et al., 1993; Holmes, 1988; Malm et al., 1992; 1994).

The fibres in a sheet of PHB are orientated in one direction which, when a sheet is rolled into a tube, can be orientated along the longitudinal axis. Based on previous experience (Curtis and Wilkinson, 1997; Whitworth et al., 1995), we hypothesized that longitudinally placed fibres would aid neuronal and glial cell growth by contact guidance and mechanical orientation. Hence, Schwann cells would align themselves along the longitudinal fibres, preferentially propagate in the longitudinal axis, and facilitate the regrowth of axons through the conduit. The aim of this study was to test this material for coapting the severed ends of a nerve, providing an end-to-end primary repair without the need for epineural sutures. The extent of the axonal regeneration and the inflammatory response to PHB were assessed morphologically up to 12 months post-operatively, and compared to standard epineural nerve repair.

MATERIALS AND METHODS

Surgical procedure

Twenty adult female cats, aged approximately 1 year, weighing 2.6 to 3.7 kg were used in the study. The animals were anaesthetised by subcutaneous medetomidin (0.1 mg/kg), intubated and ventilated with isoflurane/O_2 inhalation. Surgery was carried out under aseptic conditions and continuous monitoring. Under an operating microscope, the superficial radial nerve was exposed bilaterally on the mid-foreleg and transected. On one side, the transected nerve was enclosed in a conduit consisting of a PHB sheet wrapped around the nerve ends, leaving a gap of about 2 to 3 mm between them. The

Fig 1 Intraoperative photograph of PHB wrap-around in situ.

PHB conduits were formed from sheets with longitudinal fibre orientation. The conduits were soaked in tissue fluids in the wound for at least 5 minutes to improve their flexibility.

The formed tube was secured by one 9/0 suture at each end and sealed lengthways with Tisseel glue (Immuno AG, Vienna, Austria) (Fig 1). On the contralateral side, the nerve was sutured with 9/0 epineurial sutures. The wound was closed with resorbable 5/0 sutures. Postoperative reversal of medetomidin was achieved by the administration of atipamezol and the cats received buprenorphine as an analgesic for 3 to 5 days. At either 6 or 12 months, groups of ten animals were premedicated with medetomidin, deeply anaesthetized with intravenous thiopental (1.25 mg/kg) and intubated. Under the operating microscope, 10 mm of the superficial radial nerve at the site of the earlier transection was harvested and fixed overnight in 4% paraformaldehyde for macrophage count. Ten milimetres of the regenerated nerve, 5 mm distal to either the PHB conduit or the epineural repair, was fixed in 2.5% glutaraldehyde overnight for myelinated fibre analysis. The specimens were washed twice and then stored in phosphate buffer (0.1 M, pH 7.4) after fixation. Following the same protocol outlined above, the six superficial radial nerves from non-operated cats were used as normal control.

All experimental procedures were performed according to the European Communities Council Directive (86/609/EEC) and were approved by the Regional Committee for Ethics in Animal Experiments in Gothenberg, Sweden.

Myelinated fibre analysis

Nerve regeneration was assessed in the nerve distal to the repair site by computerized quantification of myelinated axons. The distal nerve segment was post-fixed with 1% osmium tetraoxide (Agar Scientific, Stansted, UK) in 0.1 M phosphate buffer for 1 hour at room temperature, washed with phosphate buffer and dehydrated serially through increasing concentrations of acetone. Infiltration of the specimen was initially carried out overnight with 1:1 acetone: araldite CY212 resin (Agar Scientific, Stansted, UK), followed by two changes of fresh resin and final embedding in araldite CY212 resin. The blocks were polymerized at 60°C for 18 hours. Semithin (1 μm) transverse sections were cut on a Reichert-Jung Ultracut E ultramicrotome, floated onto distilled water, collected on slides and stained with thionine blue and acridine orange to enhance the myelin contrast. Tissue analysis and all subsequent morphometric assessment were performed on coded sections without knowledge of the source. From each nerve sample, four non-adjacent fields (40 × objective) were captured from randomly chosen sections using a video camera connected to the light microscope. The captured image was automatically edited by background subtraction, image enhancement and thresholding. Monochromatic thresholding coloured the myelin red, contrasting it with the background blue and this 'thresholding' value was maintained within a narrow range for all measured sections. The measured parameters for regenerating myelinated axons were fibre diameter, myelin thickness, *g*-ratio and shape factor. The shape factor of a circle is one and measurements approximating this value indicate the regular shape of the axons. The percentage distribution of fibres according to their diameter was also charted for each group.

Macrophage counts

The repair sites from both groups were fixed overnight in 4% paraformaldehyde. After three daily changes in phosphate buffered saline (PBS) containing 15% sucrose, the specimens were blocked using OCT (Tissue-tek, Sakura, Japan). Serial longitudinal sections (15 μm) of each repair site were collected on slides coated with Vectabond (Vector Laboratories, Peterborough, UK) and allowed to dry for 4 hours at room temperature. For each sample, one slide was stained with haematoxylin and eosin and the remainder were immunostained by the indirect avidin-biotin complex peroxidase nickel enhancement procedure (Shu et al., 1988) using a monoclonal antibody to ED-1 (Serotec, Kidlington, UK, 1:600 dilution) as a marker specific for macrophages. Before a manual macrophage count, the midpoint of the repair site was identified macroscopically by bisecting the length of the section. Microscopically, in H & E and ED-1 stained sections, the exact location of the junction in both primary and PHB repairs was clearly identifiable by the presence of randomly orientated regenerating fibres and occasionally by the presence of suture holes, co-relating well to the midpoint of the repair identified macroscopically. On two randomly chosen sections from each sample, macrophages were counted in two adjacent

Table 1—Myelinated fibre analysis: results at 6 months. All values are given as mean (SEM). g-ratio = axon diameter/fibre diameter

	PHB	Epineural repair
Mean axon count	971.7 (46.61)	1029.3 (66.51)
Mean fibre diameter (μm)	5.34 (0.020)	5.30 (0.018)
Mean myelin thickness (μm)	1.55 (0.004)	1.51 (0.003)
Mean g-ratio	0.41 (0.002)	0.42 (0.002)
Mean shape factor	0.85 (0.0009)	0.83 (0.0008)

fields on either side of the midpoint across the whole width of the nerve (20 × magnification).

RESULTS

Myelinated fibre analysis

On qualitative assessment, all specimens demonstrated a mixture of regenerating axons of varying diameters. Degenerating axons, present in minimal numbers, were not counted. Tables 1 and 2 summarize the values at 6 months, 12 months and in normal non-operated nerves respectively. Statistical analysis, using the *t*-test, showed no significant difference for any measured value between the PHB and primary epineural repair groups within each time-point. One way ANOVA comparing PHB wrap-around, epineural repair and normal nerves at 12 months demonstrated a significant difference for all parameters between the PHB group and normal nerves, and similarly between epineural repair and normal groups ($P<0.05$). Comparison within the same experimental group through time (i.e. PHB or primary epineural repair at 6 and 12 months) showed a significantly greater fibre diameter in both the primary repair group (Mann-Whitney rank sum test, $P=0.003$) and the PHB group (Mann–Whitney rank sum test, $P=0.011$) (Table 3). This increase in fibre diameter with time indicates a continuing maturation of the axon. This is more clearly shown in Figures 2 and 3, where the percentage distribution of myelinated axons has been plotted according to their sizes at both time points and in normal non-operated nerves. At 6 months, the percentage pattern in PHB is similar to that of primary repair, both groups showing a unimodal distribution and a preponderance of smaller fibres with a major peak at 3 to 4 μm fibre diameter. At 12 months, the percentage pattern is again similar between the two groups. However, despite a persistent but smaller peak at 3 to 4 μm, the fibre distribution is much wider, with a definite increase in larger diameter fibres (8–12 μm diameter). The normal uninjured nerves exhibited a bimodal distribution over a range of 2 to 15 μm with peaks at 5 and 11 μm. Both operated groups also showed a decrease in mean axon count at 12 months compared with 6 months, which may indicate a die-back of axonal sprouting with continuing maturation.

Macrophage counts at the repair site

On qualitative microscopic assessment, in the PHB group the polymer remnants appeared to be decreased at 12 months compared with 6 months with disruption in fibre continuity and increased fragmentation of the polymer.

Table 4 summarizes the values of macrophage counts in the epineural repair and PHB groups at 6 and 12 months. Although there was some difference between the groups, this was not statistically significant, indicating that the foreign body reaction to PHB is comparable to that of epineural repair.

DISCUSSION

The results of this study demonstrate that the axonal regeneration, assessed by myelinated axon counts, was similar in the two experimental groups and that PHB produced an inflammatory response, gauged by the macrophage infiltration, similar to that found in primary epineural repair with no evidence of scar tissue. The conclusion is that PHB wrap-around is a good alternative to epineural repair.

Tubulization is a more "biological" approach to nerve repair whereby the neural tissue is allowed to heal by its intrinsic capacity in a closed space with minimal surgical trauma. Encasing the ends of a transected nerve in a tube with a short gap between the nerve stumps allows accumulation of locally produced neurotrophic factors. In a series of 18 patients, comparing conventional microsurgical repair with silicone tubulization in median and

Table 2—Myelinated fibre analysis: results at 12 months and in normal non-operated superficial radial nerve. All values are given as mean (SEM). $P<0.05$ for all measured parameters on comparison of epineural repair and PHB groups to normal group, one-way ANOVA test, all pairwise multiple comparison procedures (Tukey test)

	PHB	Epineural repair	Normal
Mean axon count	896 (62.62)	942.7 (51.41)	546.6 (26.31)
Mean fibre diameter (μm)	6.27 (0.026)	6.10 (0.024)	9.23 (0.145)
Mean myelin thickness (μm)	1.61 (0.004)	1.55 (0.003)	2.03 (0.026)
Mean g-ratio	0.48 (0.001)	0.49 (0.001)	0.54 (0.006)
Mean shape factor	0.83 (0.0009)	0.84 (0.0008)	0.83 (0.006)

Table 3—Comparison of fibre diameters within each experimental group. All values are given as mean (SEM) in μm

	PHB	Epineural repair
Mean fibre diameter		
6 months	5.34 (0.020)	5.30 (0.018)
12 months	6.27 (0.026)	6.10 (0.024)
P-value	0.011	0.003

Table 4—Macrophage counts. All values are given as mean (SEM)

	PHB	Epineural repair
6 months	100 (31)	72 (21)
12 months	92 (22)	87 (19)

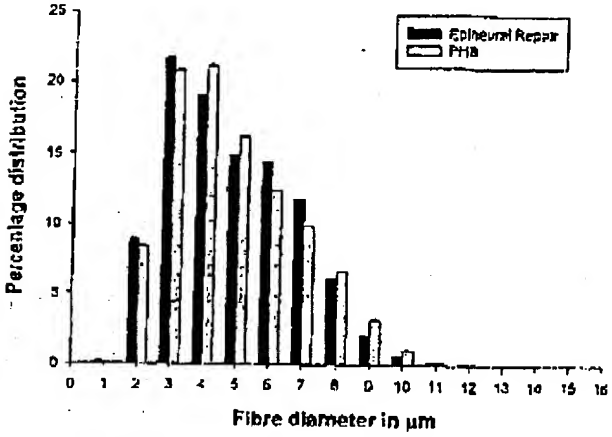

Fig 2 Size distribution of myelinated fibres at 6 months. The percentage pattern of PHB is similar to that of epineural repair, both groups showing a preponderance of smaller fibres, with a major peak at 3 to 4 μm.

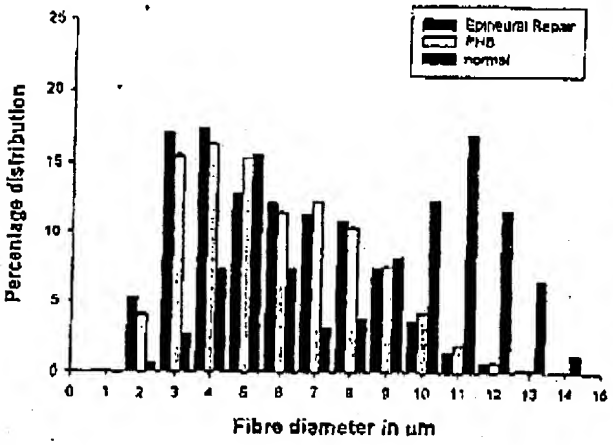

Fig 3 Size distribution of myelinated fibres at 12 months (PHB and epineural repair) and in normal non-operated superficial radial nerves. The percentage pattern in PHB is similar to that of epineural repair. The distribution of both groups is much wider with an increase in larger diameter fibres (8–12 μm). The distribution in normal nerves is bimodal with peaks at 5 and 11 μm.

ulnar nerve injuries. Lundborg et al. (1997) demonstrated no difference in the results between the two techniques. However, a second operation was necessary for removal of the silicone tube as it is non-absorbable and may cause local discomfort. Silicone entubulation can also lead to chronic nerve compression (Merle et al., 1989). As PHB is a bioabsorbable conduit, it would offer an alternative solution for the treatment of nerve injuries. As expected, in this study the polymer underwent progressive degradation, qualitatively observed at 6 and 12 months as disruption in the continuity of the fibres, fragmentation and a decrease in the polymer. The foreign body reaction to PHB, as seen by the macrophage infiltration, was minimal and similar to the reaction to sutures used in epineural repair.

Morphometric analysis of the myelinated axons within the same experimental groups showed significant differences in the fibre diameters at 12 months in comparison to 6 months, with a progressive increase in fibre diameters indicating continuing axonal maturation. The normal non-operated nerves exhibited fibres which were significantly larger at 12 months than in either of the two operated groups, implying that axonal maturation was still ongoing. However, in both PHB and epineural repair groups the axon counts at 6 months were greater than at 12 months. It is known that during regeneration after division, there is sprouting of axon collaterals with subsequent pruning back, most likely as a result of successful connections with the target organs by surviving axons (Brushart, 1993; Lundborg et al., 1994). The myelinated axon counts in normal uninjured nerves were significantly less than either the PHB or epineural repair groups at 12 months, indicating that further connections with target organs and axonal die-back continues to take place at 12 months. The size distribution pattern showed a consistent shift towards an increase in larger axons over time, with a decrease of smaller, possibly branching, fibres. A similar pattern was seen in other studies in which a synthetic conduit was used to bridge a nerve gap (den Dunnen et al., 1993; Dellon and Mackinnon, 1988).

There is a need to improve the functional results obtained from conventional microsurgical repair. Tubulization with a synthetic, bioabsorbable and relatively inert conduit such as PHB offers an alternative solution. It is easier and simpler to encase crushed, transected nerve stumps in a PHB tube, allowing the nerve to heal by its intrinsic capacity, rather than attempt to align the traumatized fascicles correctly by surgery with the risk of further damage.

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Dr G Terenghi, Blond McIndoe Laboratories, Royal Free and University College Medical School, University Department of Surgery, Royal Free Campus, Rowland Hill Street, London, NW3 2PF, UK. E-mail: blond@rmcindoeuel.demon.co.uk

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A Biodegradable Patch used as a Pericardial Substitute after Cardiac Surgery: 6- and 24-Month Evaluation with CT

*O. Duvernoy, T. Malm **, J. Ramström **, and S. Bowald **
Departments of Diagnostic Radiology, * Surgery,
** Thoracic and Cardiovascular Surgery, University Hospital, Uppsala, Sweden

Summary

Biodegradable patches made of polyhydroxybutyrate (PHB) were recently shown to limit postoperative pericardial adhesions when inserted into the pericardium in sheep. The present study was carried out to evaluate the presence of postoperative retrosternal adhesions after cardiac surgery in patients operated on with the PHB patch compared with those operated on without the insertion of such a patch. 50 patients admitted for CABG and/or valvular replacement were randomised to treatment either with pericardial closure with a patch or with the pericardium left open. In 39 of these (19 with and 20 without a PHB patch) computerised tomography was carried out six and twenty-four months postoperatively. Computerised tomography indicated a lower incidence ($p < 0.05$) of postoperative adhesions between the patch and the cardiac surface in the patch group, compared with the corresponding area in the non-patch group. A reduction of patch size in 27 % ($p = 0.003$) was also seen.

Key words

Biodegradable pericardial patch – Polyhydroxybutyrate – Pericardial substitute – Computerised tomography

Ein resorbierbarer Patch als Perikardverschluß nach herzchirurgischen Eingriffen: Ergebnisse computertomographischer Nachuntersuchungen

Durch Tierversuche an Schafen konnte kürzlich nachgewiesen werden, daß resorbierbare Patches aus Polyhydroxybutyrat (PHB) in der Lage sind, postoperative Perikardverwachsungen zu reduzieren. Wir untersuchten in der vorliegenden Studie, ob retrosternale Verwachsungen bei Patienten nach Eingriffen am Herzen durch den Einsatz dieser PHB-Patches vermieden werden können. 50 Patienten, die eine aortokoronare Bypass-Operation oder einen Klappenersatz erhielten, wurden prospektiv randomisiert und in 2 Gruppen eingeteilt. In der einen Gruppe wurde das Perikard mit einem PHB-Patch verschlossen, in der anderen wurde das Perikard offen gelassen. Bei 39 dieser Patienten (19 mit und 20 ohne PHB-Patch) erfolgte 6 und 24 Monate postoperativ eine computertomographische Nachuntersuchung. Diese Computertomogramme zeigten eine niedrigere Inzidienz ($p < 0.05$) von postoperativen Verwachsungen zwischen dem Patch und der Herzoberfläche, als dies in den korrespondierenden Arealen der Kontrollgruppe der Fall war. Eine Reduktion der Patchgröße konnte ebenfalls bei 27 % der Patienten festgestellt werden ($p = 0.003$).

Introduction

Pericardial adhesions may severely complicate reoperation by making re-entry hazardous (6). The implantation of biological or synthetic patches as pericardial substitutes has been tried with mixed results (2, 3, 4, 5, 10, 11, 12, 13, 14, 15). Recently, closure of the pericardium with absorable polyhydroxybutyrate (PHB) patches in sheep has been described (7). The pericardium regenerated completely, leaving no or discrete adhesions, and a well-defined epicardial surface was observed.

Modern imaging techniques such as computed tomography (CT) offer a non-invasive technique of investigating retrosternal adhesions in patients subjected to cardiac surgery by determining the presence or absence of fat in the retrosternal area of the heart (1). The purpose of the present study was to evaluate and compare the presence of postoperative retrosternal adhesions after cardiac surgery in patients operated on either with a PHB patch or operated on without the insertion of such a patch.

Material and Methods

Fifty patients admitted for CABG and/or valvular replacement to the Department of Thoracic and Cardiovascular Surgery, University Hospital, Uppsala, were randomised either to pericardial closure by means of a patch or to treatment where the pericardium was left open. The patches were manufactured from biodegradable polyhydroxybutyrate (PHB) provided by Astra Tech AB, Mölndal, Sweden. They were assembled from micro-filaments, packed under high pressure and at elevated temperature, thus forming a weblike structure. In a sheep model, the material was degraded by macrophages during a period of two to three years and regenerated pericardium was found to have remained (7).

The study was approved by the Ethical Committee of the University Hospital at Uppsala, Sweden.

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Operation

The patients were randomised either to closure of the pericardium by means of a PHB patch or conventional treatment where the pericardium was left wide open. The PHB patch was applied with single stitches to the edge of the native pericardium, leaving some space for pericardial blood to drain. One tube drain was placed in the pericardial cavity and one in the mediastinum. The patch was spread as smoothly as possible without any wrinkling over the heart itself.

CT

Approximately 6 and 24 months after surgery a CT study of the heart was carried out. All CT examinations were carried out on a Somatom HiQ (Siemens), using a 512×512 matrix, 2 s scan time and a soft-tissue algorithm. The heart was scannend without contrast enhancement in 4 mm contiguous slices from the level of the diaphragm to the level of the bifurcation of the pulmonary arteries. No ECG gating was employed.

The presence/absence of retrosternal fat in each scan, i.e. fat interposed between the retrosternal surface and the pericardial sac or patch, was recorded (1). In a similar manner, the epicardial fat between the cardiac surface and the pericardial sac or patch in the area corresponding to the evaluated retrosternal area was examined (Fig. 1). A scan through the mid-portion of the heart, identifiable at both the 6 and 24 month follow-up investigation, was used to calculate planimetrically the relative change in heart and patch size at this level.

Statistics

The recordings concerning the absence/presence of pericardial fat were summarised in the patch and non-patch group respectively at each scan level. A statistical comparison utilising Fisher's exact test was applied to the results of each scan level, comparing the absence/presence of epicardial fat in the patch patients with the corresponding counts in the non-patch patients. The pericardial fat was evaluated in an analogous manner. The changes in heart and patch size in the 6- and 24-month follow-up groups were compared by two-tailed paired Student's *t*-test.

Results

39 of the 50 patients in the study completed both the 6- and 24-month follow-up studies (27 male, 12 female, mean age 61.4 years; range 45 to 72 years, 19 operated upon with and 20 without a patch). Of the 11 patients not participating in both follow-up studies, one had died, one had been hospitalised due to a malignancy, two patients suffered from non-cardiac disease, making travel arrangements impossible, two patients refused to participate in the follow-up study and five patients could not be contacted. None of the follow-up patients underwent a reoperation during the observation period.

In these 39 patients, the retrosternal area, i.e. the space between the retrosternal surface and the pericardial sac or patch, displayed no statistically significant differences with regard to presence of fat between the patch and non-patch groups. On the other hand, the epicardial space in the corresponding area, i.e. the space between the patch or epicardium and the cardiac surface, was characterised by residual fat in the patch-operated group compared with the non-patch group (Figs. 1a–b, 2). In the 6-month follow-up group this difference was statistically significant in 9 CT-scan levels out of a total of 21 levels ($p < 0.05$ in 9 and of these $p < 0.01$ in 7 and $p < 0.001$ in 3). In the 24-month follow-up group the corresponding result was statistically significant in 11 levels of a total of 23 ($p < 0.01$ in 11 and of these $p < 0.001$ in 7). The change in heart volume was not statistically significant, while the mean patch volume decreased by 27% (range −84 to +14%), $p = 0.003$ (Fig. 2a, b). In some patients in the CT investigations, the pericardial thickness along the left ventricle and next to the right atrium seemed to decrease when comparing the six- and twenty-four-month investigations. However it was not possible to measure this decrease as the changes were at the limits of the CT resolution.

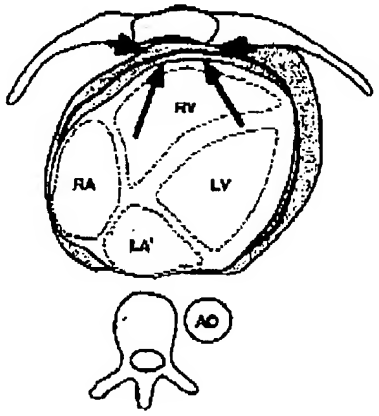

Fig. 1 Schematic drawing of axial CT scan through the mid-portion of the heart. The absence/presence of pericardial fat in the retrosternal area (short arrows) and epicardial fat (long arrows) in the corresponding area was recorded. Right ventricle (RV), right atrium (RA), left ventricle (LV), left atrium (LA), descending aorta (AO).

Discussion

Pericardial adhesions may severely complicate reoperation by increasing the risk of damaging vital structures, prolonging operation time and causing increased morbidity and mortality. Several methods have been suggested to limit postoperative pericardial adhesions. Some surgeons close the native pericardium, which is by no means always possible, as patients run a higher risk of postoperative tamponade (6).

The implantation of biological or synthetic patches as pericardial substitutes has been tried, with mixed results. Bovine material does not prevent the formation of adhesions sufficiently and calcifications of the patch have been reported (3, 9). Epicardial inflammatory reactions causing serious alterations in surface anatomy have been shown after the use of pericardial substitutes in experimental models (11). Synthetic and biological materials do not grow when implanted in individuals still growing, not to mention the risk of late infections postoperatively.

Recently, the reconstruction of the pericardium with absorbable PHB patches in sheep has been described by *Malm* et al. (7). The patch functioned as a scaffold for pericardial regeneration. Histological analysis and electron-

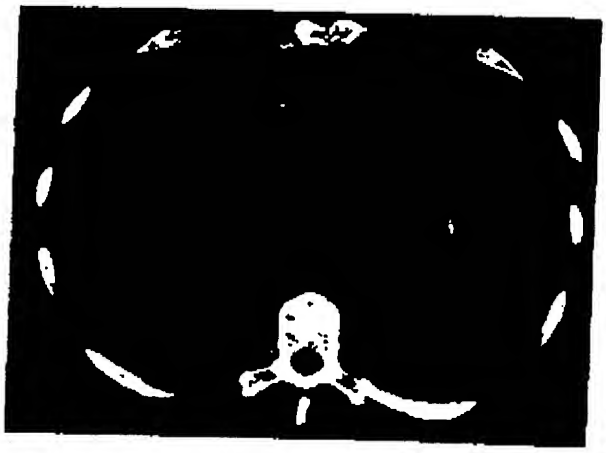

Fig. 2a Axial CT scan through the mid-portion of the heart in a patient operated by pericardial closure with a PHB patch 6 months earlier. The patch (open arrow) and the presence of fat between the patch and cardiac surface (closed arrows) is clearly seen. The fat indicates that the space between the patch and the cardiac surface is free of postoperative adhesions.

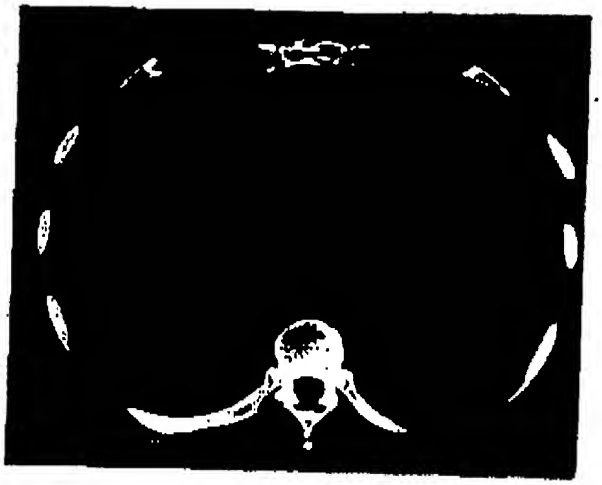

Fig. 2b The corresponding scan in the 24 month follow-up investigation, note the decrease in patch size (open arrow).

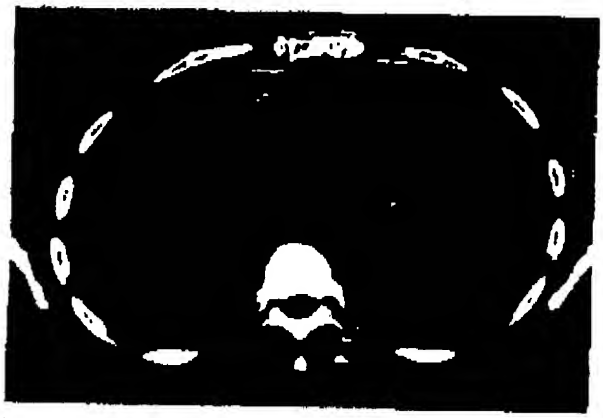

Fig. 3 Axial CT scan through the mid-portion of the heart in a patient with recent cardiac surgery without the insertion of a PHB patch. The space between the retrosternal and cardiac surface shows no fat. This indicates postoperative adhesions in this area (arrows).

microscopy studies showed regeneration of complete mesothelial layers and of dense collagen. The PHB patch was phagocytosed by macrophages and completely removed within a period of 24–30 months. The regenerated surface layer was shown to have functional properties in common with the native pericardium, such as prostacyclin production and evidence of thrombomodulin demonstrated by immunohistology (8).

The ultimate proof and evaluation of the absence/presence of postoperative adhesions can, of course, only be assessed by means of a reoperation. An accurate non-invasive method of demonstrating the postoperative status of the retrosternal space after heart surgery has still not been developed. A CT examination of the heart and pericardium can provide some indication since the presence of fat as demonstrated by CT has been shown to imply the absence of postoperative adhesions (1).

In our study, the presence of fat in the space between the patch and the cardiac surface was more common in the patch-operated group in the six- and twenty-four-month follow-up investigations compared with the corresponding area in the non-patch-operated group. This may indicate a lower incidence of postoperative adhesions between the patch and the cardiac surface in the patch group.

The patch size as revealed by the CT examinations decreased significantly, although a remnant of the patch was seen in all patients. The thickness of the native pericardium also seemed to decrease between the six- and the twenty-four-month follow-up in a few patch and non-patch patients. One explanation for this may be a residual postoperative oedema still present at the six- but not at the twenty-four-month follow-up investigation. Consequently, this has to be taken in to account when estimating the absorption of the patch by size measurement alone. Despite this, the patch does seem to decrease in size during the follow-up period.

The results after a twenty-four month follow-up period after heart surgery encourage the use of a pericardial PHB patch to reduce the incidence of adhesions between the pericardial patch and the cardiac surface.

The patch seems to decrease in size during a twenty-four month follow-up period after heart surgery.

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Dr. Olov Duvernoy
Department of Diagnostic Radiology
Akademiska Sjukhuset, Uppsala University
S-751 85 Uppsala
Sweden

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A resorbable nerve conduit as an alternative to nerve autograft in nerve gap repair

F. Hazari, M. Wiberg*, G. Johansson-Rudén†, C. Green and G. Terenghi

*Blond McIndoe Centre, East Grinstead, UK; *Department of Hand and Plastic Surgery, Umea University, Sweden and †Astra Tech, Gothenberg, Sweden*

SUMMARY. Poly-3-hydroxybutyrate (PHB) occurs within bacterial cytoplasm as granules and is available as bioabsorbable sheets. Previously, the advantage of PHB in primary repair has been investigated while in this study the same material has been used to bridge an irreducible gap. The aim was to assess the level of regeneration in PHB conduits compared to nerve autografts.

The rat sciatic nerve was exposed, a 10 mm nerve segment was resected and bridged with either an autologous nerve graft or a PHB conduit. The grafted segments were harvested up to 30 days. Immunohistochemical staining was performed and computerised quantification of penetration distance and volume of axonal regeneration was estimated by protein gene product (PGP) immunostaining and calcitonin gene-related peptide (CGRP) positive fibres. Penetration and proliferation density of Schwann cells into the conduit was measured by quantifying S-100 staining. The inflammatory response was quantified with ED-1 staining for macrophages. Antibodies to vWf provided an assessment of angiogenesis and capillary infiltration.

Percentage immunostaining for PGP in autograft and PHB groups showed a progressive increase up to 30 days with a significant linear trend with time and an increase in the volume of axonal regeneration. A similar pattern of progressive increase with time was observed with CGRP immunostaining for both groups and with S-100 in the PHB group. Good angiogenesis was present at the nerve ends and through the walls of the conduit. The results demonstrate good nerve regeneration in PHB conduits in comparison with nerve grafts. © 1999 The British Association of Plastic Surgeons

Keywords: resorbable nerve conduit, nerve autograft, rat sciatic nerve.

The use of nerve grafts to bridge irreducible nerve gaps results in sub-optimal functional results and donor site morbidity. This has led to a revival of interest in the search for a usable nerve conduit, which needs to have several properties. It should be inert, flexible, bioresorbable and inhibitory to pathological processes such as scarring and oedema, but beneficial to processes of healing and regeneration.[1] Besides being bioabsorbable, the conduit should remain in situ without degradation beyond the period of time it takes the regenerating axons to cross the gap and penetrate the distal stump.

Poly-3-hydroxybutyrate (PHB) is a storage product of bacteria, occurring within the cell cytoplasm as granules. It is available as bioabsorbable sheets, which are non-antigenic, easy to handle and have good tensile strength.[2,3] PHB undergoes hydrolytic degradation and is completely absorbed in 24–30 months.[3–5] Recently, the advantages of a PHB wrap-around in primary repair have been demonstrated.[6] In this study, the same material has been used as a conduit to bridge a 10 mm gap in the rat sciatic nerve. The aim was to assess the level of regeneration in a PHB conduit in comparison with the current standard using a nerve autograft.

Figure 1—Intraoperative photograph of PHB conduit bridging a 10 mm gap in the left sciatic nerve.

Materials and methods

Operative procedure

Thirty-six 8-week-old inbred male Lewis rats were used in the study. The animals were anaesthetised

This work was presented at the 1998 Summer meeting of the British Association of Plastic Surgeons, Colchester, UK and was awarded the John Calder Prize.

Figure 2—Unidirectional fibre orientated sheet of PHB (8 × 14 mm) rolled around a 16 G cannula to form a tube 14 mm long and with an internal diameter of 1.6 mm.

Figure 3—Intraoperative photograph of a reversed sciatic nerve autograft.

with 0.3 ml/kg of intramuscular Hypnorm (Fentanyl citrate, 0.315 mg/ml; fluanisone, 10 mg/ml; Janssen Cilag, High Wycombe, UK) and 2.5 mg/kg of intraperitoneal diazepam (Phoenix Pharmaceuticals, Gloucester, UK). All procedures were carried out in compliance with UK Home Office regulations.

Under an operating microscope (Wild, Heerbrugg, Germany), the left sciatic nerve was exposed via a gluteal muscle splitting incision. In the PHB group, a 5 mm nerve segment was resected to produce a 10 mm nerve gap after retraction of the nerve transected ends. The gap was bridged using a 14 mm PHB conduit, entubulating 2 mm of the nerve stump at each end (Fig. 1). Two 10/0 nylon sutures (Ethilon, Ethicon, Edinburgh, UK) were used to anchor the conduit to the epineurium at each end.

The conduits were formed from PHB sheets (5 × 10 cm PHB patch, Astra Tech, Gothenberg, Sweden). Rectangular pieces measuring 8 × 14 mm were cut, ensuring that the orientation of the PHB polymer fibres was along the longitudinal axis as previous experiments have shown that longitudinal orientation of PHB fibres promotes neuronal and glial growth by contact guidance. The sheets were then rolled around a 16G intravenous cannula (16G Abbocath®-T. Abbott Ireland, Sligo, Republic of Ireland), thus standardising the internal diameter of the conduits at 1.6 mm (Fig. 2), leaving adequate space for post-injury swelling of the 1 mm-diameter rat sciatic nerve. The rolled sheets were sealed longitudinally with cyanoacrylate glue (Histoacryl®, Braun Melsungen AG, Melsungen, Germany). The conduits, still rolled around the template, were pre-soaked in normal saline to saturate the polymer and ensure maximum expansion of the fibres without a reduction in the internal diameter of the conduit.

In the autograft group, a 10 mm nerve segment was resected, reversed and re-sutured in the gap with 10/0 nylon sutures (Fig. 3). Tension was avoided and atraumatic handling and correct rotational alignment were employed throughout all procedures.

Six animals from each group were sacrificed at 7, 14 and 30 days postoperatively. The repair sites (PHB conduit or nerve autograft) with a 2 mm length of proximal and distal nerve were harvested en bloc, pinned onto a card to avoid shrinkage and marked at the proximal end. Fixation was carried out in Zamboni's solution for 6 h at room temperature and then washed three times with phosphate buffered saline (PBS) containing 15% sucrose and 0.1% sodium azide.

Immunohistochemistry

The specimens were blocked in OCT compound (Tissue-tek, Sakura, Japan). The orientation of each specimen was maintained by placing a piece of rat liver next to the proximal end. Serial longitudinal sections (15 μm) were collected on slides coated with Vectabond (Vector Laboratories, Peterborough, UK) and air-dried for 4 h at room temperature. Immunohistochemical staining was carried out using the indirect avidin–biotin complex peroxidase nickel enhancement method.[7] A panel of antibody markers was employed comprising of protein gene product 9.5 (PGP 9.5, Affiniti, Mamhead, UK; dilution 1:5000) a pan-neuronal marker; calcitonin gene-related peptide (CGRP, Affiniti, Mamhead, UK; dilution 1:8000) a marker of sensory nerve fibres; S-100 (Dako A/S, Glostrup, Denmark; dilution 1:8000) an antibody marker for Schwann cells; ED-1 (Serotec Ltd, Kidlington, UK; dilution 1:600) for macrophages; and finally Von Willebrand Factor (vWF, Dako A/S, Glostrup, Denmark; dilution 1:5000) for endothelial cells.

Tissue analysis and all subsequent morphometric assessments were performed on coded sections according to a published protocol[8,9] without the knowledge of the source. A computerised image analysis system (Seescan Analytical Services, Cambridge, UK) was used throughout the study. The volume of regeneration was assessed by capturing images (magnification × 25) from two random sections, at a fixed distance of

Table 1 Maximum regeneration distance from the proximal anastomosis. Distances are given in millimetres as the mean (SEM). $n=6$

		Day 7	Day 14	Day 30
PGP	AG	7.62 (0.57)*	>10*	>10
	PHB	1.02 (0.18)	4.35 (0.37)	>10
CGRP	AG	8.15 (0.28)*	>10*	>10
	PHB	0.79 (0.17)	3.56 (0.49)	>10

*P <0.001, AG vs PHB at 7–14 days: AG = nerve autograft; PHB = conduit.

Figure 4—PGP immunostained regenerating axonal front in a PHB conduit at 7 days (× 20 objective magnification).

2 mm from the proximal repair site across the whole width of the conduit/graft, using a video camera connected to a light microscope. The captured image was automatically edited by background subtraction, image enhancement and thresholding within a narrow range for all measured frames.

The percentage of immunostaining for PGP, CGRP and S-100 between the autograft and PHB groups was then compared at the various time-points, as an indication of the number of regenerating fibres and Schwann cells. Schwann cell proliferation density distance was measured only in the PHB group, as in the autograft group, measurement of S-100 was not possible due to the inherent Schwann cell population. The penetration distance, indicative of the rate of regeneration of PGP and CGRP immunostained fibres, was measured from the proximal anastomosis (magnification × 10) in the autografts and from the beginning of the PHB polymer in the conduits. A correction factor of '−2 mm' was subtracted from all values obtained in the PHB conduit group to compensate for the entubulation of 2 mm of the proximal nerve stump into the conduit.

The inflammatory reaction was measured in both groups by manual counting of macrophages at the midpoint of the graft/conduit across the whole width of the nerve (magnification × 20) in two randomly chosen sections stained with ED-1. VWf staining was used to make a qualitative assessment of the angiogenesis and capillary infiltration.

Table 2 Percentage of immunostaining per frame 2 mm from the proximal anastomosis across the whole width of the nerve. All values are expressed as mean (SEM)

		Day 7	Day 14	Day 30
PGP	AG	3.31 (0.81)	5.84 (0.81)	7.13 (0.89)*
	PHB	3.59 (0.81)	3.63 (0.81)	4.13 (0.81)
CGRP	AG	2.72 (0.61)	3.19 (0.61)	5 (0.67)
	PHB	2.86 (0.61)	2.88 (0.61)	3.27 (0.61)
S-100	PHB	5.93 (1.004)	11.16 (0.48)	12.06 (1.27)

*P<0.001; t-test with Bonferroni correction; PGP at 30 days. AG vs PHB. AG = nerve autograft; PHB = conduit.

Results

Rate of axonal regeneration

The rate of regeneration was measured as penetration of graft/conduit by the furthermost immunostained fibres from the proximal anastomosis. Table 1 outlines the distance of regeneration for PGP and CGRP immunostained regenerating axons. At 7 days, axons in the nerve graft had crossed two-thirds of the nerve graft and in the PHB conduit had penetrated 1.02 mm of the conduit (Fig. 4). By 14 days, regenerating axons had reached the distal stump in the nerve grafts, whereas these were almost up to the halfway mark in PHB conduits. At 30 days, regenerating fibres in all animals in both groups had reached the distal anastomosis. For PGP immunostained axons, there was a significant difference in the regenerative distance between the two groups along time at 7 and 14 days (two-way ANOVA, P<0.001). This pattern was reflected in CGRP immunostained fibres (Table 1).

Amount of regeneration

The values for percentage of immunostaining of PGP and CGRP at 7, 14 and 30 days are given in Table 2. Comparison between the two groups at each of the time-points was not statistically significant except for PGP at 30 days. There was also a linear trend with time for both PGP (P=0.01) and CGRP (P=0.04) (Fig. 5), indicating that though axonal regeneration identified with the two markers is comparable between the graft types and shows a progressive increase with time, the level of regeneration in PHB conduit is slower, the difference being significant by 30 days.

Schwann cell proliferation density measured in the PHB group showed an increase with time, as shown in Table 2. Though there was a linear trend with time, the Schwann cell density appeared to reach a plateau between 14 and 30 days (Fig. 6). At this time, the axons had reached the distal stump and the levelling effect may be a reflection of the decreased impetus for Schwann cells to proliferate.

Macrophage infiltration

The inflammatory reaction to PHB was gauged by macrophage infiltration of the conduit. ED-1 immunostained macrophage counts for both groups

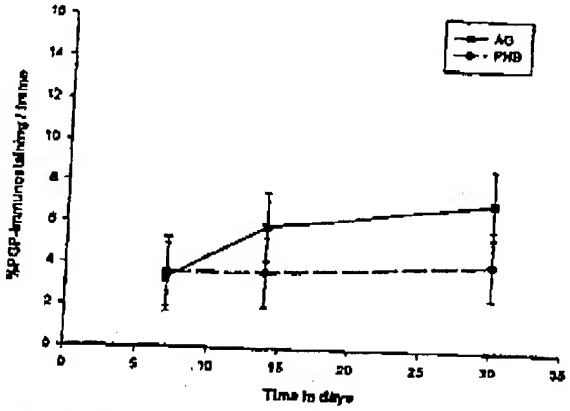

Figure 5—Amount of axonal regeneration, PHB vs nerve autograft at 7, 14 and 30 days, showing significant linear trend. $P<0.05$, two-way ANOVA.

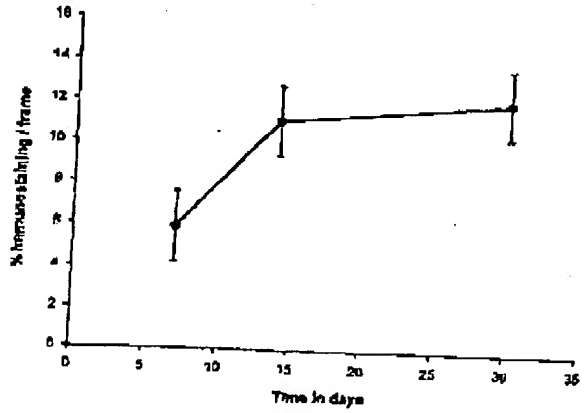

Figure 6—Schwann cell proliferation density in PHB conduit at 7, 14 and 30 days showing significant linear trend. $P<0.001$, one-way ANOVA.

are summarised in Table 3. Comparison between autograft and PHB groups showed no significant difference for macrophage counts at any of the time-points and demonstrates that the inflammatory response to PHB is not intense and was similar to that seen in nerve grafts. On the other hand, an interesting observation is that macrophage numbers appeared to increase from 7 to 14 days and then decreased to their lowest at 30 days. A two-way analysis of variance showed significantly higher numbers in both groups at 14 days compared to 30 days. This correlates well with an infiltration by macrophage scavengers at the injury site following transection, the activity increasing at 14 days and then tailing off as the regenerating axons reach the distal stump by 30 days.

Angiogenesis

A qualitative assessment of the vWf immunostained sections showed capillary penetration of the PHB conduit from either end along the proximal and distal nerve stumps and through the walls of the conduit.

Table 3 Macrophage counts. Results of AG and PHB at 7, 14 and 30 days. Macrophages were counted one frame wide at the midpoint of the conduit/graft across the whole width of the nerve at × 20 magnification. All values are given as mean (SEM). Two-way ANOVA, 14 vs 30 days

	Day 7	*Day 14*	*Day 30*
AG	15 (1.82)	20 (1.53)	12 (1.6)
PHB	16 (2.14)	18 (2.7)	11 (1.2)

AG = nerve autograft; PHB = conduit.

Discussion

The last 20 years have seen the development of many synthetic conduits and several excellent reviews are available summarising the strengths and failings of each of these.[1,10–13] Silicone has been the most widely used for experimental tubulisation and has also been applied clinically.[14] However, due to the lack of degradation of the silicone implants, it has been advocated that the next step in nerve gap repair is the use of a bioabsorbable synthetic conduit[14] which would elicit the most minimal of inflammatory reactions and would remain in situ long enough to support regeneration. Flexibility of the conduit is also necessary to enable continued protection of the regenerating nerve on initiation of mobilisation of the injured part.

PHB fulfils these criteria to a large extent. Besides being bioabsorbable, it elicits a low macrophage reaction comparable to that of a nerve graft. This was also observed in our previous study wherein a PHB wrap-around sheath to join the divided nerve was compared to primary epineural nerve repair.[6] In this study, macrophage numbers in both groups are similar with no significant difference, increasing from 7 to 14 days, this increase correlating well with the period of intense phagocytic activity during Wallerian degeneration[15] and then decreasing significantly by 30 days when the axons have reached the distal stump. Clinically, a low inflammatory response is desirable to prevent adhesions to surrounding structures, in particular to tendons in hand injuries.

The rate of regeneration measured by the regenerative distance into the conduit from the proximal repair site, though not fully equivalent to that of a nerve graft, still appears to be comparable for both PGP and CGRP immunostained fibres. Regenerating axons grow into the first part of the PHB conduit by 7 days, come up to the halfway mark by 14 days and reach the distal stump by 30 days, and it is important to note that there was no failure of regeneration in any of the implanted conduits. This time-scale is in keeping with observations made when other biodegradable conduits such as fibronectin mats have been used.[7] Despite good results obtained with fibronectin mats, the clinical application of this type of conduit may be difficult as it undergoes rapid reabsorption in 3 weeks and has inherent problems associated with pooled plasma from which it is manufactured.[3,7] A longer reabsorption time, as with PHB, ensures that the regenerating and maturing nerve is able to withstand the stress of mobilisation.

The volume of axonal regeneration quantified by the percentage of PGP and CGRP immunostaining appears to be comparable between the two graft types. There is a progressive increase in the amount of regeneration with time, although the difference between nerve grafts and PHB becomes statistically significant by 30 days. The regeneration pattern in the PHB conduit is reflected by the Schwann cell proliferation density, which levels off between 14 and 30 days. This result correlates well with the slowing down of the rate of Schwann cell proliferation demonstrated after the initial burst during Wallerian degeneration, which is thought to be signalled by axonal contact.[16,17]

In conclusion, this study demonstrates good axonal regeneration in PHB conduits with a low level of inflammatory infiltration. This is a good indication of the suitability of PHB as a resorbable synthetic conduit for nerve gap repair. We are aware that the rate and amount of regeneration in PHB conduits does not fully compare with that observed in a nerve graft, but this difference is due to the cellular elements inherently present in nerve grafts which aid regeneration. It is well known that de-differentiated Schwann cells synthesise neurotrophic factors[18] which are known to promote nerve regeneration when administered exogenously to an injured nerve.[8,19] It is tempting to speculate that the level of regeneration in a PHB conduit may be further improved by the addition of growth factors, bringing nearer the concept of a composite conduit to obtain optimal nerve regeneration.

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The Authors

Anita Hazari MBBS, FRCS. Research Registrar in Plastic Surgery Blond McIndoe Centre, Queen Victoria Hospital, East Grinstead RH19 3DZ, UK.

Mikael Wiberg MD, PhD. Consultant Hand and Plastic Surgeon Department of Hand and Plastic Surgery, University Hospital, Umeå S-90185, Sweden.

Gunilla Johansson-Rudén MSc(Chem Eng). Associate Director Research & Development, Astra Tech AB, PO Box 14, Mölndal S-43121, Sweden.

Giorgio Terenghi PhD, MRCPath, Deputy Director and Head of Nerve Regeneration Group

Colin Green DSc, FRCPath, FRCS(Hon), Director Blond McIndoe Centre, Queen Victoria Hospital, East Grinstead RH19 3DZ, UK.

Correspondence to Dr G. Terenghi.

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